

NAD Metabolism in *Vibrio cholerae*

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Extracts of *Vibrio cholerae* were assayed for various enzymatic activities associated with pyridine nucleotide cycle metabolism. The activities measured include NAD glycohydrolase, nicotinamide deamidase, nicotinamide mononucleotide deamidase, and nicotinic acid phosphoribosyltransferase. The results obtained demonstrate the existence in *V. cholerae* of a five-membered pyridine nucleotide cycle and the potential for a four-membered pyridine nucleotide cycle. The data presented also suggest that most of the NAD glycohydrolase in *V. cholerae* extracts is not directly related to cholera toxin.

Vibrio cholerae produces a potent enterotoxin, cholera toxin, whose role in the pathogenesis of cholera has been well established (5, 6, 14). The effect of this toxin on eucaryotic cells has been extensively studied (2, 7, 10). The toxin uses NAD as a substrate in the ADP ribosylation and activation of adenylate cyclase (2, 10, 16). NAD glycohydrolase and ADP-ribosylating activities are associated with the A₁ peptide of cholera toxin (18), which indicates that cholera toxin is similar to other NAD-dependent bacterial toxins such as diphtheria toxin (12), *Pseudomonas aeruginosa* exotoxin A (3), and *Escherichia coli* heat-labile enterotoxin (17).

Despite extensive research on the mode of action of this toxin, nothing is known about its biological role within the bacterial cell. Fernandes et al. (4) have presented data which suggest that transport of the enterotoxin occurs across the inner and outer membranes during toxin secretion. They discovered a 29,000-dalton protein with NAD glycohydrolase activity associated with the inner membrane and a 22,000-dalton protein with similar activity associated with the outer membrane. They propose that the 29,000-dalton protein is processed to form a 22,000-dalton toxin precursor protein during transport. The NAD glycohydrolase associated with the inner membrane was capable of transferring ADP ribose to another inner membrane protein which possessed a relative molecular weight of 44,000. Consequently, they proposed that the NAD glycohydrolase and ADP ribosyltransferase enzymes associated with the membrane of *V. cholerae* are toxin related and play a regulatory role in the bacterial cell.

These and other studies show that NAD metabolism is of central importance to this toxin and its protomer forms. It might be predicted, for example, that mutants defective in toxin

production should possess lower NAD glycohydrolase levels. However, nothing is known about normal NAD metabolism of *V. cholerae*. The purpose of this investigation, therefore, was to examine pyridine nucleotide cycle metabolism in *V. cholerae*.

The bacterial strains used throughout this study are listed in Table 1. All strains were grown either in the basal E medium of Vogel and Bonner (20), supplemented with 0.4% glucose, or in syncase medium (7). Cultures were grown at 37°C in a New Brunswick gyratory incubator at 240 rpm.

Cells were grown in 1 liter of E medium with a 5% inoculum from an overnight culture. When the culture reached the late exponential phase (optical density at 540 nm = 0.6), the cells were harvested by centrifugation at 16,000 × g for 15 min, washed twice with 10 mM Tris (pH 7.6) containing 1 mM MgCl₂, 2 mM tosyl-1-lysine chloromethyl ketone, and 2 mM tosylamide-2-phenylethyl chloromethyl ketone, and recentrifuged. The pellet was stored at -70°C until needed.

Extracts were then prepared by suspending 2 g of cells (wet weight) in 10 ml of 100 mM potassium phosphate buffer (pH 7.6) containing 2 mM tosylamide-2-phenylethyl chloromethyl ketone and tosyl-1-lysine chloromethyl ketone. Sonication was performed in an ice-salt-water bath with a Branson Sonifier operated in 20-s bursts with 40-s cooling until the optical density at 640 nm reached 10% of the initial optical density. The extract was clarified by centrifugation at 27,000 × g for 15 min. Glycerol was then added to a final concentration of 15% (wt/vol).

The standard reaction mixture for NAD glycohydrolase and NAD pyrophosphatase contained 10 μmol of sodium phosphate buffer (pH 7.5), 360 nmol of NAD containing 0.2 μCi of [¹⁴C]car-

TABLE 1. Bacterial strains used

<i>V. cholerae</i> strain	Phenotype	Source and reference
569B	Hypertoxigenic	P. Fernandes
CA401	Wild type	C. Parker (1)
FA18	Asp ⁻	C. Parker (1)
FA64	Prototrophic	C. Parker (1)
FA86	Prototrophic	C. Parker (1)
FA131	Met ⁻	C. Parker (1)
3083	Prototrophic, wild type	R. Finkelstein (11)
Texas Star SR	Prototrophic, toxin A negative	R. Finkelstein (11)

bonyl-labeled NAD or nicotinamide [U - 14 C]adenine dinucleotide, and 10 μ l of the enzyme preparation in a final volume of 100 μ l. Reactions were terminated by the addition of 25 μ l of 30% cold perchloric acid and neutralized with potassium hydroxide, and the potassium perchlorate precipitate was removed by centrifugation. Ten microliters was cochromatographed with unlabeled NAD, nicotinamide mononucleotide (NMN) deamidase, and nicotinamide (NA_m) deamidase on 1-in. (ca. 2.54-cm) strips of Whatman no. 1 filter paper. Chromatography was done with either a 1 M ammonium acetate-95% ethanol (3:7) solvent system adjusted to pH 5.0 with concentrated HCl or an isobutyric acid-concentrated ammonium hydroxide-water (66:1:33) solvent system. After drying, the strips were cut into segments corresponding to the unlabeled reference markers and counted in a Beckman 9000 liquid scintillation counter. NMN deamidase, nicotinic acid phosphoribosyltransferase, and NA_m deamidase were all assayed as previously described (8). Protein determinations were made as described by Lowry et al. (15).

Previous work from this laboratory with *Salmonella typhimurium* has provided evidence for three NAD recycling pathways (8, 13). Therefore, crude extracts of *V. cholerae* 569B were assayed for several pyridine nucleotide cycle components at various pH values to determine which of these pathways may function. Distinct evidence for a five-membered pyridine nucleotide cycle was found in that NAD glycohydrolase, NA_m deamidase, and nicotinic acid phosphoribosyltransferase activities were detected. Crude extracts of CA401 examined for these enzymes yielded similar results. Complete recycling of nicotinic acid to NAD was found when *V. cholerae* 569B was grown in the presence of [14 C]nicotinic acid. The majority (80%) of intracellular label was found as NAD, although other intermediates were detected. The evidence suggests that NAD synthesis occurred via the classic Preiss and Handler pathway (9).

Evidence was also found which suggested the presence of an alternate recycling pathway. NMN deamidase activity was readily demon-

strable in crude extracts of 569B (Table 2) and CA401. Molecular sieve chromatography with Sephadex G-150 revealed that NMN deamidase and NA_m deamidase were two separate enzymatic activities with native molecular weights of 43,000 and 35,000, respectively. The presence of a distinct NMN deamidase suggests a four-membered recycling pathway similar to that reported in several other organisms (9). However, an essential component of this cycle, NAD pyrophosphatase, was not detected, presumably because of the high level of NAD glycohydrolase activity present.

Since any proposed role for cholera toxin in the physiology of *V. cholerae* would necessarily involve NAD metabolism, several toxigenic and virulence-deficient mutants were examined for NAD glycohydrolase, NAD pyrophosphatase, NMN deamidase, and NA_m deamidase activities. We predicted that some of the toxigenic mutants should have drastically reduced NAD glycohydrolase activities if the massive wild-type glycohydrolase activity observed was due primarily to A subunit precursors. Also, with a reduced glycohydrolase background, NAD pyrophosphatase activity might be demonstrable. The results (Tables 2 and 3) did not confirm these predictions. None of the mutant strains tested possessed NAD glycohydrolase activities significantly lower than that of the parent, CA401. A hypertoxigenic strain, 569B, also did not yield significantly higher glycohydrolase activity. Strain FA86, although apparently normal in toxin production, possessed approximately twice the parental glycohydrolase activity. Of the other activities tested, NA_m deamidase levels did not differ significantly among the various strains, but FA64 extracts consistently yielded NMN deamidase activities almost 2.5 times higher than that of the parent. The significance of the apparent alterations in NAD metabolism relative to the reduced virulence of these strains is not clear. Unfortunately, none of the mutants exhibited NAD pyrophosphatase activity. This was not unexpected, however, since all of the extracts still exhibited very high NAD glycohydrolase levels.

The results suggested that toxin production

TABLE 2. Comparison of pyridine nucleotide cycle activities in various strains of *V. cholerae*^a

Strain	Sp act ^b			Reduction in toxin activity ^c (fold)
	NAD glycohydrolase	NAm deamidase	NMN deamidase	
CA401	132 ± 18.5	19 ± 2.1	69 ± 8.5	None
FA18	93 ± 9.5	18 ± 3.5	56 ± 7.2	64
FA64	99 ± 12.0	22 ± 3.1	166 ± 9.1	16
FA86	257 ± 21.1	16 ± 2.0	66 ± 8.6	None
FA131	130 ± 17.0	24 ± 3.2	69 ± 6.6	512
569B	118 ± 9.2	15 ± 3.5	80 ± 7.5	

^a Cell-free extracts were prepared from cells grown to the late log phase in minimal medium supplemented with 0.4% glucose and the appropriate auxotrophic requirements.

^b NAD glycohydrolase activity (pH 7.5) is expressed as nanomoles of NAm formed per minute per milligram. NAm deamidase (pH 6.5) and NMN deamidase (pH 9.0) activities are expressed as nanomoles of product formed per hour per milligram. All values represent the average of three separate extractions ± the standard deviation.

^c Data obtained from Baselski et al. (1).

and intracellular NAD glycohydrolase activity were not directly related. It is not known whether the toxin-deficient strains FA18, FA64, and FA134 are simply toxin release mutants or actually toxin deficient. Thus, it was necessary to examine a true toxin-negative strain. The Texas Star SR mutant isolated by Honda and Finkelstein is a strain which produces no detectable A subunit in culture filtrates or in cell-free extracts as determined by erythrocyte adenylate cyclase assays (11). Consequently, if the NAD glycohydrolase activity measured in the other strains was due solely to unreleased toxin, then Texas Star should have minimal glycohydrolase activity. Texas Star grew poorly in the minimal medium employed in this study. Therefore, Texas Star, 3083 opaque, 3083 translucent, and CA401 strains were grown in syncase medium for comparative purposes before extract preparation. The results (Table 3) were completely unexpected. Texas Star possessed more than 2.5 times the glycohydrolase activity of CA401, grown under similar conditions, and slightly more than its parent, 3083. This finding suggests that there are NAD glycohydrolases in *V. cholerae* unrelated to toxin. NMN deamidase activities for both 3083 and Texas Star were somewhat lower than that for CA401 (70%). NAm deamidase,

however, was 2.5 times greater in Texas Star than in CA401 and approximately 2 times greater than in 3083. These variations appear to be statistically significant based upon the standard deviation values obtained with multiple extractions. Once again, no NAD pyrophosphatase activity was observed.

NAD glycohydrolase activity measured in the various toxin mutants appears to reflect isoenzymes of NAD glycohydrolase other than cholera toxin. Purified cholera toxin (Sigma Chemical Co.) possessed an NAD glycohydrolase activity of 18 nmol/min per milligram, much lower than that observed in cell-free extracts. This finding reinforced the theory that much of the NAD glycohydrolase activity measured in cell-free extracts was not directly related to unreleased cholera toxin. Nichols et al. (19) have suggested that cholera toxin is synthesized intracellularly in a precursor form. Their studies uncovered a 52,000-dalton protein that immunoprecipitated with anti-subunit A antibody and a 45,000-dalton polypeptide that immunoprecipitated with both anti-subunit A and anti-subunit B antibodies. The smaller protein is presumably the precursor of the A and B subunits of cholera toxin. However, the relationship of the larger polypeptide to either the 45,000-dalton protein

TABLE 3. Pyridine nucleotide cycle activities in Texas Star SR extracts

Strain	Sp act ^a		
	NAD glycohydrolase	NMN deamidase	NAm deamidase
CA401	90 ± 13.5	57.1 ± 6.4	29.2 ± 3.4
3083 (translucent)	188 ± 18.1	39.4 ± 2.1	36.2 ± 5.3
3083 (opaque) ^b	175 ± 21.5	36.6 ± 2.6	26.9 ± 6.5
Texas Star SR	240 ± 22.3	40.1 ± 3.2	73.4 ± 8.1

^a Cell-free extracts were prepared from cells grown to the late log phase in syncase medium. NAD glycohydrolase activity is expressed as nanomoles per minute per milligram of protein. NMN and NAm deamidase activities are expressed as nanomoles per hour per milligram of protein. All values represent the average of three separate extractions ± the standard deviation.

^b 3083 opaque is a hypotoxigenic variant of 3083 translucent. 3083 translucent is the parent of Texas Star SR.

or cholera toxin itself is not known. Nevertheless, their data do support the concept of NAD glycohydrolase isoenzymes in *V. cholerae*.

We thank R. Finkelstein and P. Kasvinsky for their helpful discussions and critical readings of the manuscript.

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